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Occludin as a possible determinant of tight junction perm ability in endothelial cells

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SUMMARY

Endothelial cells provide a crucial interface between blood and tissue environments. Free diffusion of substances across endothelia is prevented by the endothelial tight junction, the permeability of which varies enormously depending on tissue. Endothelial cells of the blood-brain barrier possess tight junctions of severely limited permeability, whereas those of non-neural tissue are considerably leakier, but the molecular basis for this difference is not clear. Occludin is a major transmembrane protein localizing at the tight junction. In this study, we show, by immunocytochemistry, that occludin is present at high levels and is distributed continuously at cell-cell contacts in brain endothelial cells. In contrast, endothelial cells of non-neural tissue have a much lower expression of occludin, which is distributed in a discontinuous fashion at cell-cell contacts. The apparent differences in occludin expression levels were directly confirmed by immunoblotting. The differences in occludin protein were reflected at the message level, suggesting transcriptional regulation of expression. We also show that occludin expression is developmentally regulated, being low in rat brain endothelial cells at p st-natal day 8 but clearly detectable at post-natal day 70. Our data indicate that regulation of occludin expression may be a crucial determinant of the tight junction permeability properties of endothelial cells in different tissues.

Key words: Tight junction, Occludin, Endothelial cell, Blood-brain barrier

INTRODUCTION

One of the most important properties of endothelial cells and epithelial cells, attributable to the intercellular tight junction, is the creation of a paracellular barrier for ions, proteins and other solutes (Gumbiner, 1987; Rubin, 1992; Schneeberger and Lynch, 1992; Citi, 1993; Anderson and Van Itallie, 1995). The barrier plays an important role not only in normal physiology, to maintain tissue environments, but also in pathological situations, such as vasogenic oedema and inflammation.

From the perspective of the tightness of the barrier, endothelial cells differ considerably in phenotype. Brain endothelial cells, which form the blood-brain barrier (BBB), are coupled by tight junctions of extremely low permeability, that are, indeed, more like those of epithelial barriers. In contrast, endothelial cells in non-neural tissues are coupled by much leakier tight junctions (Rubin, 1991). The molecular basis responsible for these different junctional phenotypes and the regulatory mechanisms that control tight junctions are not yet clear. Astrocyte-endothelial cell interactions have been suggested to contribute to differentiation of brain endothelial cells and hence to the formation of the BBB (Janzer and Raff, 1987). Further, brain endothelial cell tight junctions are subject to regulation by a variety of cytoplasmic signalling systems (Staddon and Rubin, 1996). However, complete information concerning the role of astrocytes and intracellular events is still lacking.

Another factor which can affect the paracellular barrier formed by tight junctions in endothelial cells is the integrity of adherens junctions (Rutten et al., 1987), as is also the case for epithelial cells (Gumbiner and Simons, 1986). Disruption of the adherens junction by removal of extracellular Ca^{2+} leads to the opening of the tight junction. The Ca^{2+}-sensitive components of the adherens junction are cadherins (Takeichi, 1991), single pass transmembrane proteins which associate via their cytoplasmic domain with α-, β- and γ-catenins (Ozawa et al., 1989) and p120, a tyrosine kinase substrate (Reynolds et al., 1994; Shibamoto et al., 1995; Staddon et al., 1995).

It also seems likely that tight junction permeability is regulated by components of the tight junction itself. Several such components have been identified, mainly using epithelial cells. These include ZO-1 (Stevenson et al., 1986), ZO-2 (Gumbiner et al., 1991), cingulin (Citi et al., 1988), 7H6 antigen (Zhong et al., 1993) and symplekin (Keon et al., 1996), but all are peripherally localized cytoplasmic proteins. p130 was also identified as a protein associating somehow with ZO-1 and ZO-2 (Balda et al., 1993). So far, however, occludin is the only integral membrane protein exclusively localized at the tight junction (Furuse et al., 1993). Occludin is known to

associate with ZO-1 (Furuse et al., 1994). Tight junctions therefore seem to consist of at least an occludin:ZO-1:ZO-2:p130 complex. Exactly how the other tight junction proteins interact with occludin, if at all, is not yet clear.

Occludin was clearly seen to be localized at endothelial cell junctions in microvessels in chicken brain and was more faintly detected in endothelial cells in microvessels of heart muscle (Furuse et al., 1993). However, because of the lack of available probes, there has been no information about the expression and localization of occludin in mammalian endothelial cells. Now mammalian occludin and its cDNA have been identified (Ando-Akatsuka et al., 1996), and a rat monoclonal anti-mouse occludin antibody, as well as a rabbit polyclonal anti-mouse occludin antibody, that recognizes mammalian occludin has now been described (Saitou et al., 1997).

In this study, we have examined occludin expression in brain endothelial cells, in tissue sections and in cell culture, and have compared its expression with that in endothelial cells from non-neural tissues. Our study indicates that occludin expression differs vastly in different endothelial cells, perhaps by transcriptional regulation. The effects of some of the known tight junction permeability modulators on occludin expression levels has also been investigated. Finally, we have investigated developmental changes in occludin expression in brain endothelial cells.

MATERIALS AND METHODS

Reagents

All tissue culture materials were from Gibco (Paisley, UK) except for plasma-derived bovine serum (PDBS) which was from Advanced Protein Products Ltd (Brierley Hill, Midlands, UK). Gel electrophoresis reagents were from Bio-Rad (Hemel Hempstead, Hertfordshire, UK). Other reagents used were of the highest grade commercially available.

Antibodies

The mouse monoclonal antibody against ZO-1 (T8-754) was raised against a rat adherens junction fraction (Itoh et al., 1991), and the rat monoclonal antibody (MRK) and the rabbit polyclonal antibody were both raised against glutathione S-transferase (GST)-mouse occludin fusion protein (Saitou et al., 1997). The mouse monoclonal anti-β-catenin antibody was from Transduction Laboratories Ltd (Lexington, KY). The rabbit polyclonal anti-collagen IV was from Biogenesis (Poole, UK). Primary antibodies were detected using FITC-conjugated goat anti-rat IgG, TRITC-conjugated goat anti-mouse IgG and TRITC-conjugated goat anti-rabbit IgG, which were from Jackson Immunoresearch Laboratories Ltd (West Grove, PA).

To verify the specificity of the rabbit polyclonal anti-occludin polyclonal antibody, 0.6 ml antiserum containing 1 mg protein was absorbed either by 4 mg of GST or 6.8 mg of GST-mouse occludin bound to glutathione-Sepharose 4B following a protocol available from Pharmacia Biotech (Uppsala, Sweden).

Cell culture and transendothelial electrical resistance measurements

Primary cultures of porcine brain endothelial cells and astrocyte-conditioned medium (ACM) were prepared essentially as described by Rubin et al. (1991). Culture medium for brain endothelial cells was composed of 50% astrocyte-conditioned medium and 50% DME containing 10% PDBS, 125 μg/ml heparin, antibiotics and glutamine. For transcellular electrical resistance measurements with a pair of fixed, current-passing, voltage-measuring electrodes (World Precision Instruments, Hertfordshire, UK), brain endothelial cells were grown on collagen-coated, 0.4 μm polycarbonate Transwell filters (Costar Corp., Cambridge, MA, USA). To examine the effects of ACM, porcine brain endothelial cells were cultured in either medium for brain endothelial cells containing ACM or the same medium in which ACM was replaced by DME with 10% PDBS. Also, porcine brain endothelial cells were treated with 8-(4-chlorophenylthio) cyclic AMP (CPT-cAMP) and lysophosphatidic acid (LPA) either alone or together, as follows. After attaining confluence in culture medium containing ACM, cells were treated with either 250 μM CPT-cAMP for 60 minutes or 10 μM LPA for 30 minutes. When treated in combination, cells were incubated initially with CPT-cAMP for 30 minutes and then with LPA for a further 30 minutes. Control cells were cultured in medium containing neither CPT-cAMP nor LPA.

Primary cultures of porcine aortic endothelial cells were prepared essentially as described by Schwartz (1978). LLC-PK$_1$ cells were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK) and cultured at 37°C under an atmosphere of 5% CO_2 in DME containing 10% FCS.

Immunocytochemistry

All procedures were at room temperature. Cells were fixed in 3% paraformaldehyde in PBS for 15 minutes. After fixation, the cells were rinsed and permeabilized by incubation with 0.2% Triton X-100 in PBS for 15 minutes. After rinsing, the cells were blocked in 1% BSA in PBS for 15 minutes and incubated with primary antibody for 60 minutes. After rinsing, cells were incubated for 60 minutes with a 1:100 dilution of FITC-conjugated goat anti-rat IgG, TRITC-conjugated goat anti-mouse IgG or TRITC-conjugated goat anti-rabbit IgG in PBS containing 1% BSA. Following a final rinse, the cells were mounted with Citifluor (Citifluor Products, Canterbury, UK) and examined using a Nikon Microphot-FXA fluorescence microscope with ×20 and ×40 objectives. Photographs were taken using Kodak T-MAX film (400 ASA).

For preparing cryosections from guinea pig and rat, tissues were frozen using liquid nitrogen. Tissue blocks were mounted in Tissue Tek (R. Lamb, London, UK), and sections of 10 μm thickness were cut using a cryostat (Bright Instrument Company Ltd, Cambridge, UK), mounted on glass slides and air dried for 30 minutes. Sections were stained using the same method as that described for cultured cells but without permeabilization. In rat tongue tissue, reactivity of the FITC-conjugated goat anti-rat IgG with endogenous IgG was detected, so we used tongue from guinea pig for immunostaining with rat anti-occludin antibody. For comparative purposes, guinea pig brain and sciatic nerve were also studied. For the developmental study using rat tissue, it was established that endogenous IgG was not a problem as in all cases the staining was completely dependent on the presence of the primary antibody.

Immunoelectron microscopy on freeze-fractured replicas

Details of the immunoelectron microscopic procedure for examining freeze-fracture replicas were as described by Fujimoto (1995). Porcine brain endothelial cells were cultured on a collagen coated dish and then removed by scraping. The cells were pelleted and then quickly frozen by being slammed against a pure copper block cooled by liquid helium gas (Heuser et al., 1979). The frozen samples were fractured at −110°C and platinum-shadowed unidirectionally at an angle of 45° in a Balzers Freeze Etching System (BAF 400T; Balzers Corp., Hudson, NH). The samples were immersed in sample lysis buffer containing 2.5% SDS, 10 mM Tris-HCl and 0.6 M sucrose, pH 8.2, for 12 hours at room temperature. Then the replicas floating off the samples were washed with PBS. Under these conditions, integral membrane proteins were captured by the replicas, and their cytoplasmic domains were accessible to antibodies. The replicas were incubated with the rabbit polyclonal anti-occludin antibody for 60 minutes and then rinsed with PBS followed by incubation with the goat anti-rabbit IgG coupled to 10 nm gold (Amersham International plc, Buckinghamshire, UK). The samples were rinsed with PBS,

picked up on Formvar-filmed grids and examined using a JEOL 122EX electron microscope at an accelerating voltage of 80 kV.

Gel electrophoresis and immunoblotting

After rinsing the cells briefly with PBS, whole-cell lysates were prepared by extraction into SDS sample buffer (Laemmli, 1970) followed by heating at 100°C for 5 minutes. The protein content in the lysates was measured by a dye-binding protein assay based on the method of Winterbourne (1986). Proteins were electrophoretically resolved by one-dimensional SDS-PAGE (10% polyacrylamide) based on the method of Laemmli (1970). The gels were then equilibrated in transfer buffer containing 48 mM Tris, 39 mM glycine, 0.03% SDS and 20% methanol. Proteins were electrophoretically transferred to nitrocellulose filters followed by Ponceau S staining. To detect occludin, an alkaline phosphatase method was used. Filters were blocked in 5% non-fat dried milk in TBS (TBSM) and incubated with the rabbit polyclonal anti-occludin antibody at 4°C for 16 hours. After washing with TBS containing 0.05% Tween-20 (TBST), the filters were incubated with a 1:500 dilution of biotinylated anti-rabbit Ig (Amersham International plc) in TBSM for 60 minutes at room temperature. After washing with TBST, the filters were incubated with a 1:3,000 dilution of a streptavidin-alkaline phosphatase conjugate (Amersham International plc) in TBSM for 30 minutes at room temperature. Following a final wash with TBST, alkaline phosphatase was detected using 0.4 mM nitro blue tetrazolium and 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate in buffer containing 100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 50 mM $MgCl_2$, followed by quenching with water.

To detect ZO-1 and β-catenin, horseradish peroxidase detection was used. PBS, instead of TBS, was used in the washing and blocking buffers. A 1:500 dilution of biotinylated anti-mouse Ig, as secondary antibody, and a 1:2,000 dilution of horseradish peroxidase-streptavidin conjugate, as tertiary antibody, were used. Horseradish peroxidase was detected by luminography using SuperSignal chemiluminescent substrate (Pierce and Warriner UK Ltd, Chester, UK). Integrated band density was determined by densitometry using a Bio-Rad model GS-670 imaging densitometer (Bio-Rad).

Partial cDNA cloning and northern blotting

For the partial cDNA cloning of porcine occludin, PCR was performed using a porcine lung λgt11 cDNA library (Clontech Laboratories, Inc., Palo Alto, CA) as template with the oligonucleotides 5′TATGAGACAGACTACACAACTGGCGGCGAGTCC3′ and 5′ATCATAGTCTCCAACCATCTTCTTGATGTG3′ as primers (see Ando-Akatsuka et al., 1996). A cDNA fragment obtained by PCR was subcloned into pBluescript SK(−) and sequenced using the 7-deaza Sequenase Version Deoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).

Total RNA from cultured cells was isolated according to the method of Chomczynski and Sacchi (1987), and poly(A)$^+$ RNA was purified using a Poly(A) Quick mRNA Isolation Kit (Stratagene, La Jolla, CA). About 5 μg of poly(A)$^+$ RNA from each cell type and an RNA ladder (Boehringer Mannheim UK Ltd, East Sussex, UK) as molecular size standards were resolved using a formaldehyde/agarose (1%) gel and then transferred to a nitrocellulose filter. The PCR-generated occludin cDNA fragment and the insert of mouse skeletal muscle β-actin cDNA clone I (Stratagene) were labelled with [α-^{32}P]dCTP (Amersham International plc) using a Prime-It II Random Primer Labelling Kit (Stratagene) and then used as probes. Hybridization with the occludin probe was carried out under conditions of high stringency (50% formamide, 5× Denhardt's solution, 5× SSC, 0.5% SDS and 100 μg/ml of denatured salmon sperm DNA at 42°C). After detection of occludin mRNA by autoradiography, the filter was stripped by washing it twice with 0.1× SSC containing 0.1% SDS at 68°C for 30 minutes. The filter was then reprobed for β-actin mRNA under the same conditions.

RESULTS

Occludin in microvessels from neural and non-neural tissue

The expression of occludin in endothelial cells has been reported only for microvessels of chicken brain and heart muscle, as determined by immunofluorescence microscopy (Furuse et al., 1993). It has yet to be determined whether mammalian brain endothelial cells express occludin and whether occludin expression levels differ in various mammalian endothelial cell types in vivo. To determine occludin expression in mammalian brain microvessels and to compare occludin expression with that of ZO-1, we examined vascular tissue of neural and non-neural origin in adult guinea pig by immunofluorescence microscopy. In brain endothelial cells, we observed strong junctional staining with antibodies recognizing either occludin (Fig. 1A) or ZO-1 (Fig. 1D). Similar results were obtained with peripheral nerve microvessels (Fig. 1B,E). In contrast, occludin showed extremely weak staining in endothelial cells from non-neural tissue such as tongue (Fig. 1C), whereas staining of ZO-1 was observed at a similar intensity to that seen in brain microvessels (Fig. 1F). Thus occludin expression levels seemed to differ considerably between the different types of endothelial cells, whereas those of ZO-1 were more similar.

The localization of occludin in cultured endothelial cells

In order to further investigate the expression and localization of occludin in endothelial cells of brain and non-neural tissue, we prepared primary cultures of endothelial cells from pig brain and aorta. Immunofluorescence microscopy demonstrated that brain endothelial cells in culture clearly expressed occludin in a continuous fashion at cell-cell contacts which colocalized with ZO-1 (Fig. 2A,B). In contrast, occludin was hardly detectable at junctions in aortic endothelial cells (Fig. 2D; longer exposure: Fig. 2G). The intensity of staining with the anti-ZO-1 antibody was as strong as that seen in the brain endothelial cells, but, in contrast, there was discontinuous staining at cell-cell contacts (Fig. 2E). As ZO-1 localized at cell borders with different staining patterns, continuous and discontinuous, in each cell type, we also compared the localization of β-catenin, a cadherin-associated component of adherens junctions. Like that of ZO-1, β-catenin staining was also continuous at cell borders in brain endothelial cells (Fig. 2C), but discontinuous in aortic endothelial cells (Fig. 2F). In both cell types, the intensity of staining was similar. These results indicate that brain endothelial cells possess continuous junctional structures at cell-cell borders, whereas those of aortic endothelial cells are fragmented.

Furthermore, to confirm that occludin is a component of tight junction strands, a key structure observed by freeze-fracture electron microscopy, brain endothelial cells were freeze-fractured. A freeze-fracture replica in which tight junction strands were seen (Fig. 3A) was labelled with the rabbit polyclonal anti-occludin antibody and detected with the goat anti-rabbit IgG coupled to 10 nm gold (Fig. 3B). In the replica immunolabelled with the anti-occludin antibody, gold particles accumulated at tight junction strands, verifying occludin to be a component of tight junction strands in brain endothelial cells.

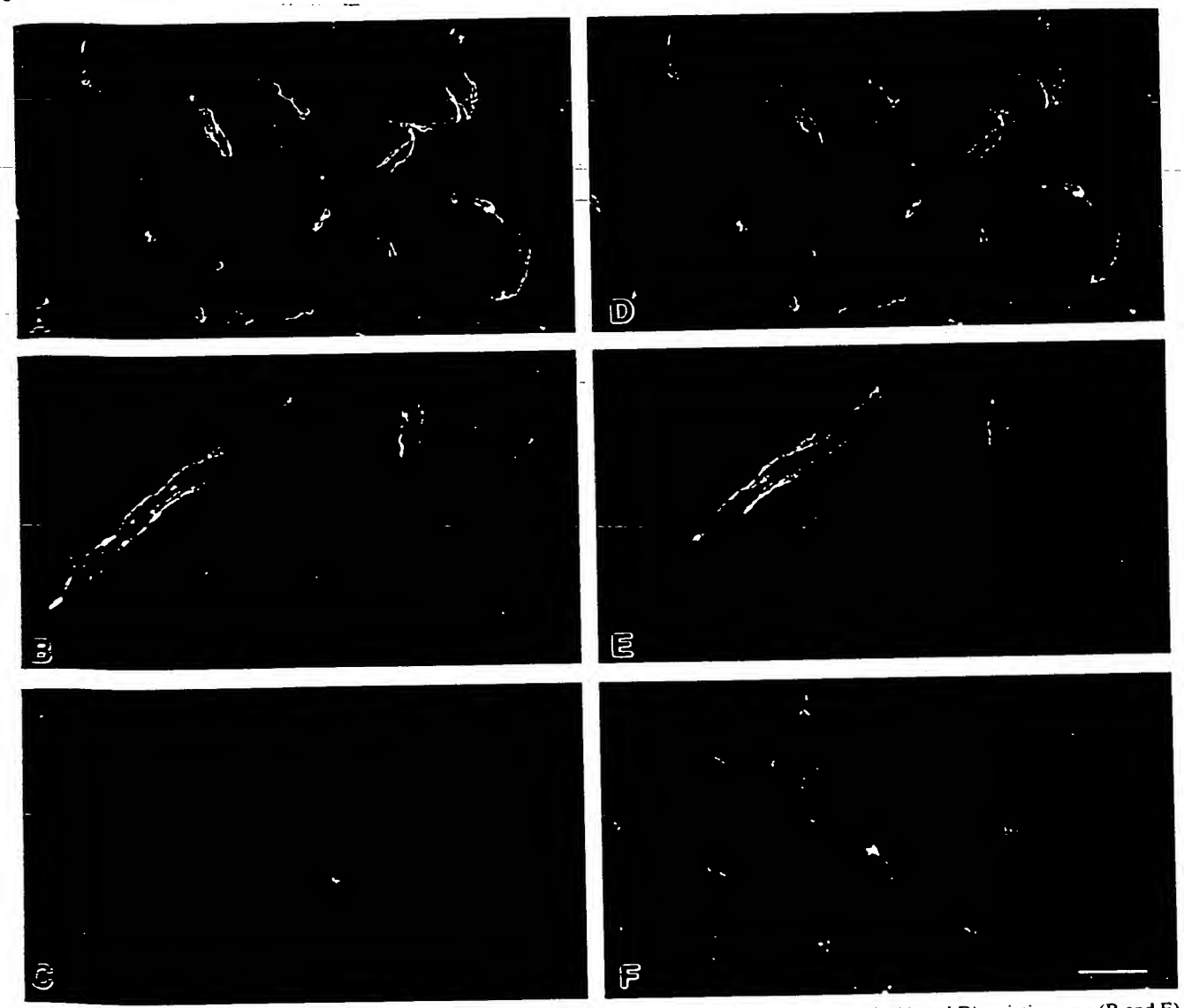

Fig. 1. Subcellular distribution of occludin (A,B,C) and ZO-1 (D,E,F) in frozen sections of guinea pig brain (A and D), sciatic nerve (B and E) and tongue (C and F). The frozen sections were doubly stained with the rat monoclonal anti-occludin antibody (MOC37) and the mouse anti-ZO-1 antibody (T8-754), and detected with FITC-conjugated goat anti-rat IgG and TRITC-conjugated goat anti-mouse IgG, respectively. It was verified that each secondary antibody was absolutely specific for its designated species of primary antibody and that neither spillover of FITC into the TRITC channel nor the reverse was observed. Also, in these cases, endogenous IgG in the tissue was not detected by the secondary antibodies (results not shown). In brain and peripheral nerve, occludin and ZO-1 colocalize at interendothelial junctions of microvessels. On the other hand, in tongue, while ZO-1 is clearly present at endothelial cell junctions, occludin is hardly detectable. Also, junctional staining of endothelial cells of brain microvessels was seen with the rabbit polyclonal anti-occludin antibody. Staining was still seen with the antibody absorbed by GST but not with the antibody absorbed by GST-mouse occludin (data not shown). The localization of occludin and ZO-1 in microvessels were confirmed by colabelling sections with an antibody against collagen IV, a known marker for endothelial cell basement membrane (data not shown; see Staddon et al., 1995). Exposure times were the same for A-F. Bar, 20 μm.

Occludin protein measurement in cultured endothelial cells

Next, by immunoblotting (Fig. 4A), we examined the expression of occludin in both types of endothelial cells and, for comparison, in the porcine epithelial cell line LLC-PK$_1$, which has well-developed tight junctions (Mullen et al., 1992). Whole-cell lysates were prepared from the three cell types and, for each, the same amount of total cellular protein was resolved by one-dimensional gel electrophoresis. To compare occludin expression quantitatively, a dilution series of a whole-cell lysate from brain endothelial cells was prepared and analyzed on the same gel. Immunoblotting was performed using the anti-

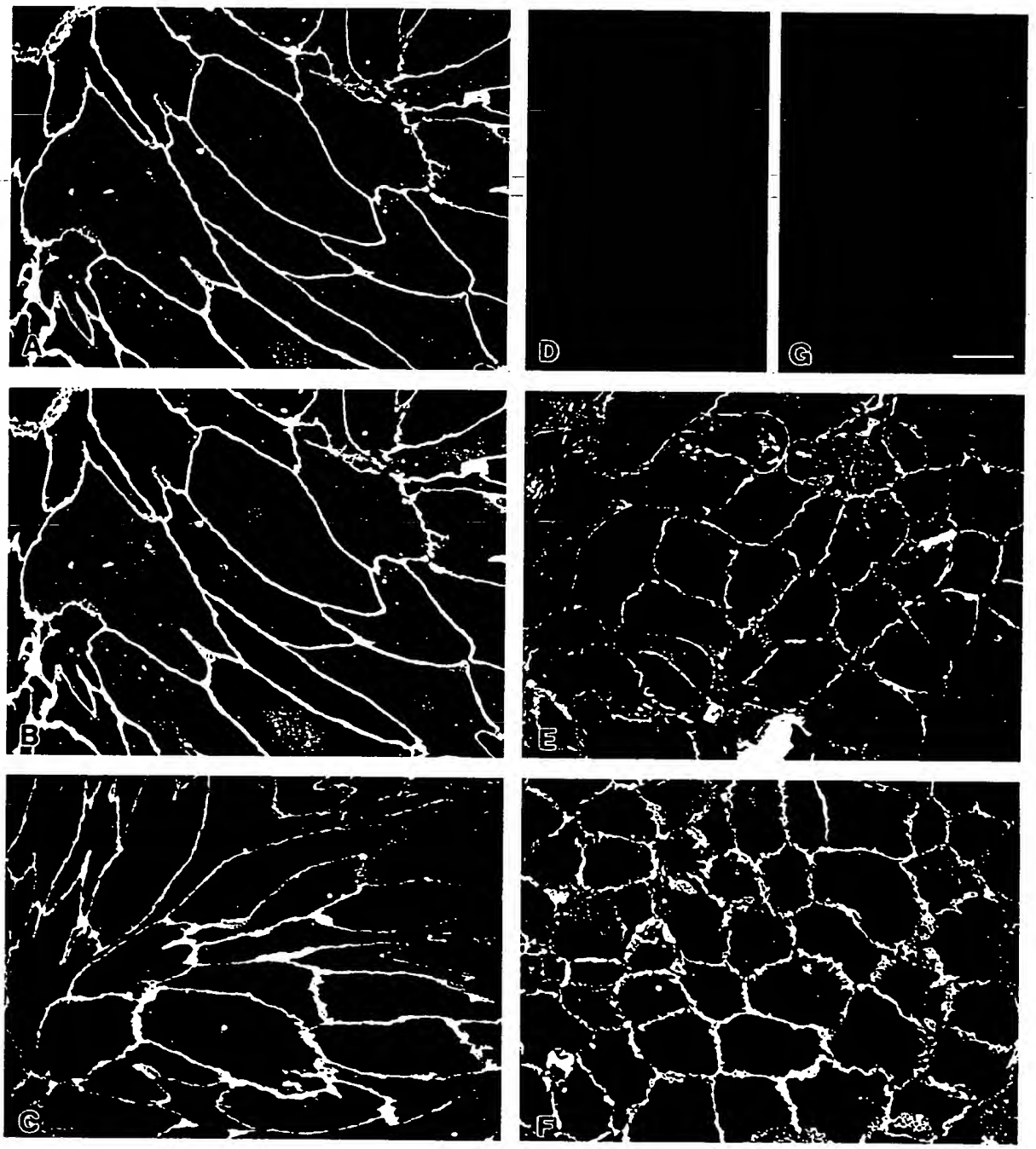

Fig. 2. Localization of occludin (A,D,G), ZO-1 (B and E) and β-catenin (C and F) in porcine brain (A,B,C) and aortic endothelial cells (D-G). Cells were colabelled with the rat monoclonal anti-occludin antibody and the mouse anti-ZO-1 antibody, or labelled with the mouse anti-β-catenin antibody. Exposure times for A-F were constant but that for G was three times longer. In brain endothelial cells, occludin and ZO-1 colocalize at cell-cell contacts in a continuous fashion (A and B). β-Catenin also localizes at cell-cell contacts continuously (C). In contrast, occludin is hardly detectable in aortic endothelial cells (D), even at a longer exposure time (G). ZO-1 and β-catenin localize at cell-cell contacts of aortic cells in a discontinuous fashion and the staining intensity is similar to that in brain endothelial cells (E and F). Bar, 20 μm.

occludin antibody and, then, the intensities of the occludin immunoreactivity were quantified by densitometry (Fig. 4B,C,D).

In brain endothelial cells and LLC-PK$_1$ cells, occludin was clearly detected as several bands migrating at about 58 kDa, whereas in aortic endothelial cells occludin was more weakly

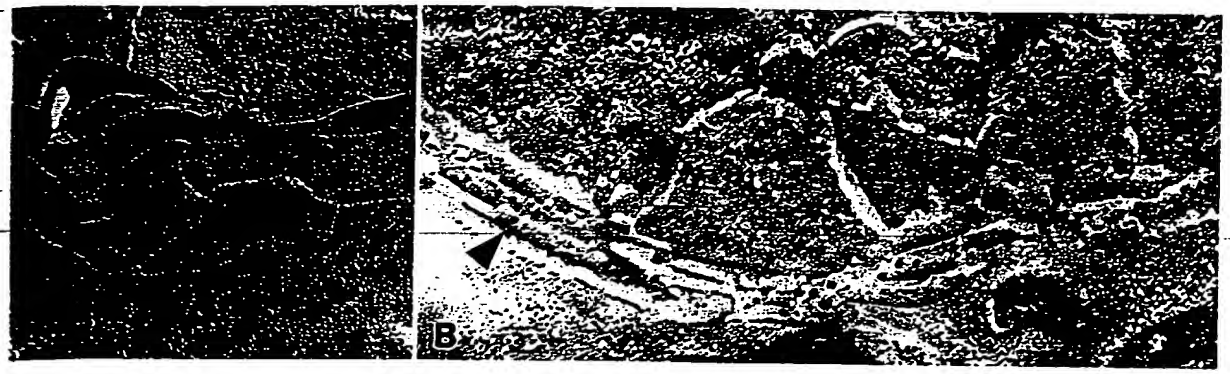

Fig. 3. Immunoelectron microscopy on freeze-fractured replicas of porcine brain endothelial cells with the rabbit polyclonal anti-occludin antibody. Tight junction strands (arrowhead) were seen in a freeze-fractured replica (A). A replica was labelled with the rabbit anti-occludin antibody and detected with the goat anti-rabbit IgG coupled to 10 nm gold (B). Gold particles accumulated at tight junction strands (arrowhead). Bars, 0.1 μm.

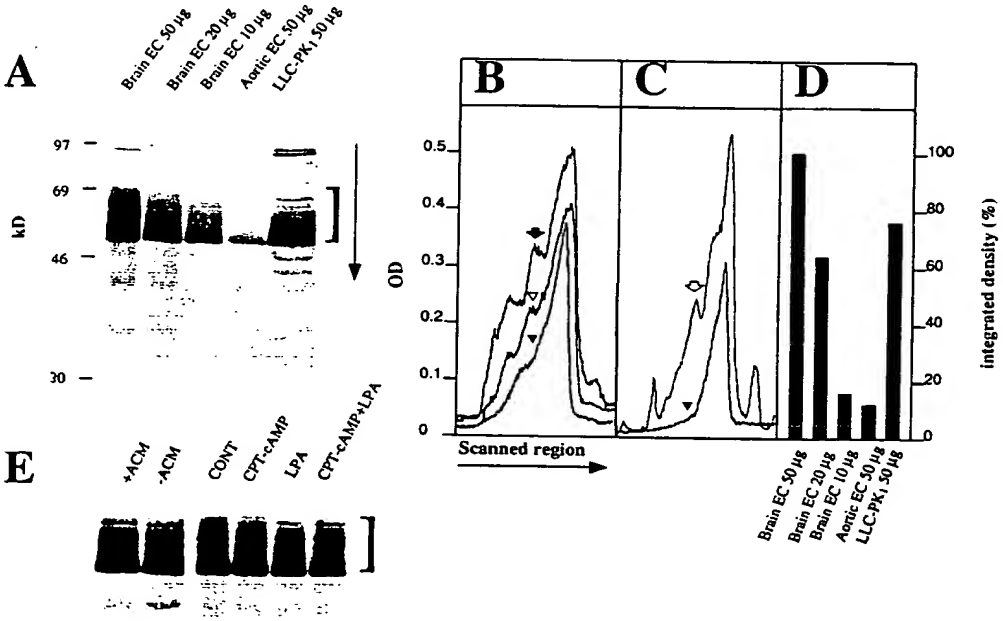

Fig. 4. Occludin expression in brain endothelial cells, aortic endothelial cells and LLC-PK$_1$ cells. Whole-cell lysates from porcine brain endothelial cells (Brain EC), porcine aortic endothelial cells (Aortic EC) and LLC-PK$_1$ cells containing 50 μg of total protein and a dilution series of whole-cell lysate from brain endothelial cells were resolved by one-dimensional gels and immunoblotted using the rabbit polyclonal anti-occludin antibody (A). The bars indicate the migration of molecular mass standards (kDa). The rabbit polyclonal anti-occludin antibody was chosen rather than the rat monoclonal anti-occludin antibody because it gives superior results in immunoblots. The bands indicated by a bracket in A were detected by the antibody absorbed with GST but not by the antibody absorbed by GST-mouse occludin, which were considered to be occludin immunoreactive. The regions in A (arrow) were scanned by densitometry, showing quantitatively the intensities of occludin bands in each lysate (B,C,D). Scans in B are shown for 50 μg (black arrow), 20 μg (open arrowhead) and 10 μg (black arrowhead) lysates from brain EC. In C, scans for aortic EC (black arrowhead) and LLC-PK$_1$ (open arrow) are indicated. The integrated density of each band was normalized to that of 50 μg lysate from the brain EC and shown in D. Quantification of immunoblots of lysates from brain EC containing less than 10 μg of total protein was considered unreliable because of the band distortion that occurred during electrophoresis with this low amount of protein. (E) The effects of tight junction permeability modulators on occludin expression levels in brain endothelial cells. Whole-cell lysates were prepared from porcine brain endothelial cells cultured with (+ACM) or without ACM (−ACM; see Materials and Methods), resolved by one-dimensional gels and immunoblotted (E, left). Also, porcine brain endothelial cells were treated with CPT-cAMP and LPA either alone or together. Control cells (CONT) were cultured in medium containing neither CPT-cAMP nor LPA. To verify biological activity, transendothelial electrical resistance measurements were performed (n=10). The resistances of cells treated with CPT-cAMP, LPA and both together were 1.8±0.2, 0.6±0.1 and 0.8±0.3 (mean ± s.d.) -fold of that of the control value, respectively. Whole-cell lysates prepared from the above cells were resolved by one-dimensional gels and immunoblotted (E, right). Occludin expression levels in brain endothelial cells were not affected by any of these treatments. The bands corresponding to occludin are indicated by the bracket.

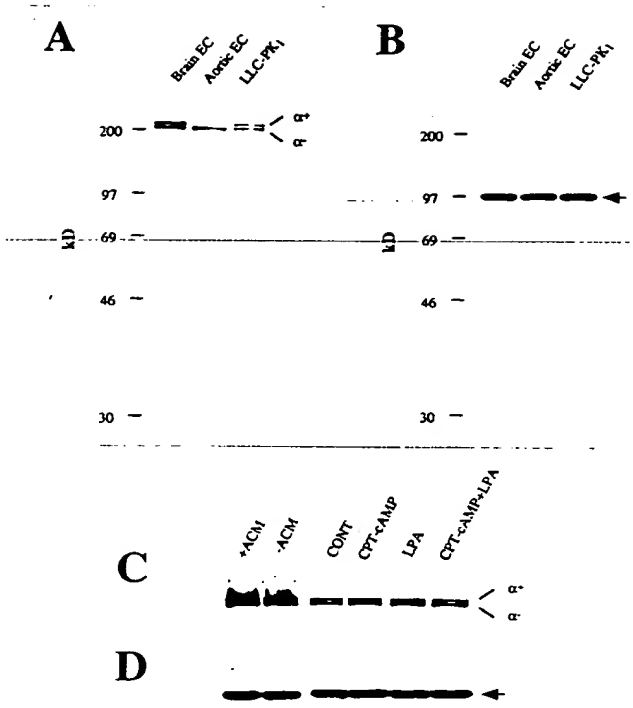

Fig. 5. ZO-1 and β-catenin expression in brain endothelial cells, aortic endothelial cells and LLC-PK$_1$ cells. Whole cell lysates prepared from the three cell types were resolved by one-dimensional gels and immunoblotted with antibodies recognizing either ZO-1 or β-catenin. In porcine brain endothelial cells (Brain EC) and LLC-PK$_1$ cells, two bands of ZO-1 immunoreactivity that probably correspond to the α^+ and α^- isoforms of ZO-1 were detected, whereas, in porcine aortic endothelial cells (Aortic EC), only the faster-migrating band was observed (A). The expression level of β-catenin was similar in the three cell types (B). As described in the legend to Fig. 4, brain endothelial cells were treated with different permeability modulators and the effects on ZO-1 (C) and β-catenin (D) were examined. The bands corresponding to ZO-1 and β-catenin (arrows) are indicated.

observed. In brain endothelial cells, the fastest migrating occludin bands were the most intense, but, in addition, more slowly-migrating, fainter bands were detected. In LLC-PK$_1$ cells, the fastest-migrating occludin band had the same molecular mass and was present in similar intensity to that in brain endothelial cells. However, in these cells, the more slowly-migrating bands were weaker in intensity than those from brain endothelial cells. The integrated density of the immunoreactive occludin band in 10 μg lysate from brain endothelial cells was slightly higher than that of 50 μg lysate from aortic endothelial cells, indicating at least a 5-fold greater amount of occludin protein in the extract from brain endothelial cells compared to that from aortic endothelial cells. Occludin expression levels therefore appear to be related to tight junction phenotype, being high in brain endothelial cells and low in endothelial cells of non-neural tissue.

To further investigate a potential role for occludin in regulating paracellular barrier properties, we tested the effects of some of known tight junction permeability modulators on occludin expression levels in porcine brain endothelial cells. ACM and CPT-cAMP, a cyclic AMP analogue, have been shown to induce tight junctions of lower permeability in cultured brain endothelial cells of bovine (Rubin et al., 1991) and porcine origin (Schulze et al., 1997). On the other hand, LPA has been found to cause a rapid, reversible and dose-dependent increase in the paracellular permeability of brain endothelial cells, even in the presence of CPT-cAMP (Schulze et al., 1997). We prepared whole-cell lysates from cells treated with these reagents (see Materials and Methods). The same amount of total cellular protein for each was resolved by one-dimensional gels and then analyzed by immunobloting with the rabbit polyclonal anti-occludin antibody (Fig. 4E). Expression levels of occludin did not appear to change in response to ACM, CPT-cAMP and LPA. We also examined the effects of longer (16 hours) treatment with CPT-cAMP, and again the occludin expression did not change (data not shown). Immunofluorescence microscopy confirmed that the localization of occludin did not appear to be affected in response to any of these treatments (data not shown). Thus, although brain endothelial cells clearly express high levels of occludin, this, as far as we have examined, does not appear to be affected by several tight junction permeability modulators.

ZO-1 and β-catenin in cultured endothelial cells

Since brain endothelial cells are characterized by high occludin expression, for comparison we also investigated the expression of ZO-1 and β-catenin in both types of endothelial cells and in LLC-PK$_1$ cells. As reported previously, the mouse monoclonal anti-rat ZO-1 antibody, T8-754, recognizes two bands around 220 kDa in immunoblots of an isolated rat adherens junction fraction (Itoh et al., 1991, 1993). These two bands probably correspond to the α^+ and α^- isoforms of ZO-1 (Willott et al., 1992). Using T8-754, immunoblots of extracts derived from brain endothelial cells and LLC-PK$_1$ cells revealed two similarly intense bands around 220 kDa (Fig. 5A). Also, in aortic endothelial cells, in spite of the difficulty in detecting occludin, the faster-migrating band of ZO-1 was clearly observed although the slower-migrating band could not be seen. The adherens junction-associated protein β-catenin was detected as a 97 kDa protein at a similar intensity in each cell type (Fig. 5B). Thus, the banding pattern of ZO-1 in the different types of endothelial cells appeared to differ, whereas the content of β-catenin was similar.

We also investigated the effects of the tight junction permeability modulators ACM, CPT-cAMP and LPA on the expression levels of ZO-1 and β-catenin. The expression levels of ZO-1 and β-catenin did not change in response to any of these treatments (Fig. 5C,D). Also, the localization of ZO-1 or β-catenin was apparently not affected (data not shown). These data indicate that tight junction permeability modulators do not affect the expression levels of ZO-1 or β-catenin, or their localization.

Occludin messenger RNA in endothelial cells

We then explored the possibility that higher occludin expression in brain endothelial cells may be due to transcriptional regulation. First, we isolated a porcine occludin partial cDNA by PCR using a porcine lung λgt11 cDNA library as template and two primers that were previously reported (Ando-

Akatsuka et al., 1996). A 363-base pair product was amplified. The fragment was clearly a partial cDNA of porcine occludin as the deduced amino acid sequence of the fragment showed high homology (~90% identity) to the sequences of human, mouse and dog occludin (Fig. 6A).

Then we examined the message levels of occludin in porcine brain endothelial cells, porcine aortic endothelial cells and LLC-PK$_1$ cells by northern blotting using the PCR-generated fragment as a probe. As shown in Fig. 6B, an intense band of 2.4 kb was observed in mRNA from brain endothelial cells and LLC-PK$_1$ cells, whereas occludin mRNA was not detectable in aortic endothelial cells. Reprobing for β-actin mRNA clearly indicated equal loading of mRNA from each cell type. These results suggest that the expression of occludin may be transcriptionally regulated, although effects on message stability cannot be excluded. Also, the fainter bands around 3.5 kb could indicate the possible existence of alternatively-spliced forms of occludin in brain endothelial cells, and similar results have been obtained with epithelial cells (Saitou et al., 1997).

Occludin and the maturation of brain capillaries

Tight junction permeability in brain endothelial cells, which is considered to be a crucial determinant of the BBB, has been suggested to vary during development, although the developmental timing of the formation and maturation of BBB in vivo is still controversial (Farrell and Risau, 1994). Also, so far, little information about potential developmental changes in the expression of molecular components of the tight junction has been available. To evaluate changes in expression of occludin and ZO-1 in brain endothelial cells in vivo, we examined rat brain at post-natal day 8 (P8) and day 70 (P70) using immunofluorescence microscopy (Fig. 7). Secondary antibody reactivity with endogenous IgG in rat brain tissue was not a problem as all staining shown was completely primary antibody-dependent.

Endothelial cells in brain microvessels at P70 clearly showed occludin expression (Fig. 7E), whereas occludin was hardly detected in brain microvessels at P8 (Fig. 7A). In contrast, ZO-1 could be observed at junctions in brain microvessels both at P8 (Fig. 7B) and at P70 (Fig. 7F). Further highlighting this difference in expression in endothelial cells, occludin and ZO-1 could be found in epithelial cells of the choroid plexus equally at P8 (arrows in Fig. 7C,D) and P70 (arrows in Fig. 7G,H). Also, ventricular ependymal cells, which had low levels of occludin, but had high ZO-1 expression both in P8 (arrowheads in Fig. 7C,D) and P70 brain (arrowheads in Fig. 7G,H), are considered to have leakier tight junctions than endothelial cells (Risau and Wolburg, 1990). Thus, occludin expression is clearly upregulated in brain endothelial cells during development, raising the possibility that this correlates with development of the BBB.

DISCUSSION

The composition and amounts of the protein components of the tight junction in endothelial cells with different paracellular

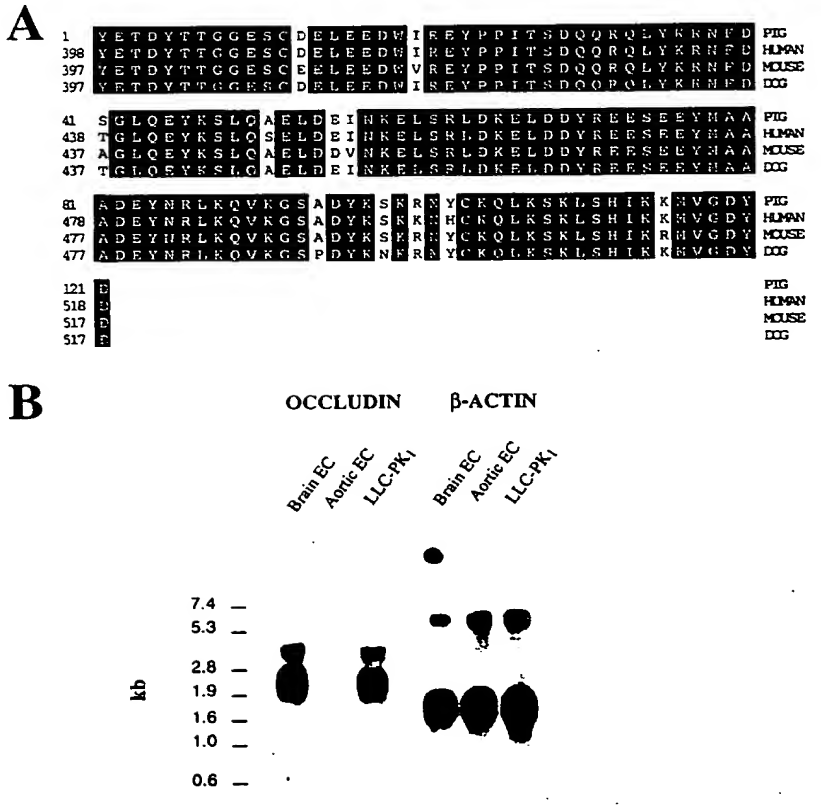

Fig. 6. Porcine occludin partial cDNA and northern blot. (A) Comparison of the deduced amino acid sequence from the porcine occludin partial cDNA with those of human, mouse and dog as analyzed by the MegAlign program. Conserved amino acids between all species are indicated by black boxes. The porcine sequence data are available from EMBL/GenBank/DDBJ under accession number U79554. (B) Northern blot of poly(A)$^+$ RNA (5 μg/lane) isolated from porcine brain endothelial cells (Brain EC), porcine aortic endothelial cells (Aortic EC) and LLC-PK$_1$ cells. The blot was hybridized under high stringency conditions with the porcine occludin probe, then stripped and reprobed for mRNA encoding β-actin. The bars on the left indicate the migration of RNA standards (kb).

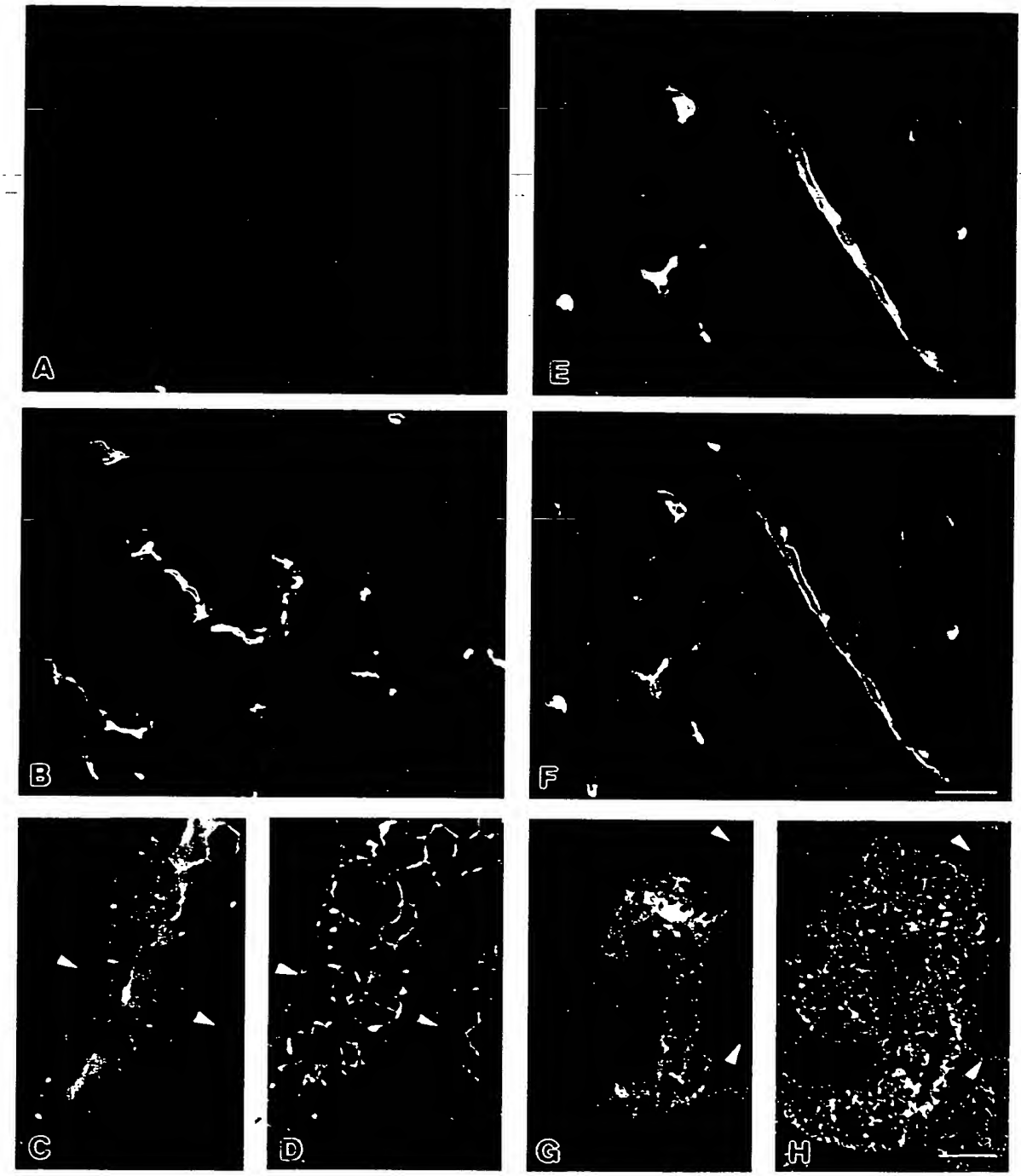

Fig. 7. Expression and localization of occludin (A,C,E,G) and ZO-1 (B,D,F,H) in brain microvessels (A,B,E,F), choroid plexus epithelial cells (arrows in C,D,G,H) and ventricular ependymal cells (arrowheads in C,D,G,H) of rat at postnatal day 8 (P8) (A,B,C,D) and 70 (P70) (E,F,G,H). Frozen sections of rat brain at P8 and P70 were colabelled with the rat anti-occludin antibody (MOC37) and the mouse anti-ZO-1 antibody (T8-754) followed by the detection with FITC-conjugated goat anti-rat IgG and TRITC-conjugated goat anti-mouse IgG, respectively. Exposure times were constant in A-H. The intensity of occludin staining in brain microvessels at P70 (E) is higher than at P8 (A), whereas that of ZO-1 is constant at both stages (B and F). Choroid plexus epithelial cells at both stages had similar levels of occludin (arrows in C and G) and ZO-1 (arrows in D and H), and both were localized at all junctions. In ventricular ependymal cells of both stages, ZO-1 was easily detected at junctions (arrowheads in D and H), but occludin staining was hardly detectable (arrowheads in C and G). Bars: 10 μm (A-F); 20 μm (G and H).

barrier properties have not been fully investigated. In particular, information about integral membrane proteins of these junctions has been lacking. So far, occludin is the only integral membrane protein known to be localized at the tight junction, and, although it was initially cloned from the chicken (Furuse et al., 1993), it clearly has mammalian homologues (Ando-Akatsuka et al., 1996). The availability of mammalian occludin cDNA and antibodies that recognize the mammalian protein enabled us to study occludin not only in vivo but also in cell culture. In this study, to investigate tight junctions in endothelial cells, we focused on occludin, comparing its expression and localization with those of ZO-1, a well-studied tight junction associated protein (Stevenson et al., 1986; Anderson et al., 1988; Itoh et al., 1991, 1993; Willott et al., 1993).

Endothelial cells in brain microvessels possess tight junctions of remarkably low permeability, which are to a large extent responsible for forming and maintaining the BBB in vivo (Risau and Wolburg, 1990; Rubin, 1991). Our immunofluorescence microscopy clearly demonstrated occludin expression and colocalization with ZO-1 in endothelial cells of guinea pig brain microvessels. We carried out immunoelectron microscopy of freeze-fractured replicas and demonstrated occludin to be a component of tight junction strands in cultured brain endothelial cells (Fig. 3). Fujimoto (1995) presented data consistent with ours that occludin was a component of tight junction strands in epithelial cells. In contrast, occludin was hardly detectable in endothelial cells of non-neural tissue such as tongue (Fig. 1).

In culture, brain endothelial cells also clearly expressed occludin at cell-cell contacts, and colocalization with ZO-1 was again observed (Fig. 2). The continuous staining of occludin, ZO-1 and β-catenin at cell borders in brain endothelial cells indicates that these cells are circumscribed by continuous junctional structures, as would be expected for a tight paracellular barrier. In contrast, in aortic endothelial cells, occludin was hardly detectable. ZO-1 and β-catenin were expressed, but displayed a discontinuous staining at cell borders, corresponding to a fragmented junctional structure that may be responsible for a leaky barrier.

Our observations using immunocytochemistry were also reflected in immunoblot analysis, where brain endothelial cells were characterized by high occludin expression when compared with aortic endothelial cells (Fig. 4A). Recently Balda et al. (1996) and McCarthy et al. (1996) have demonstrated that overexpression of chicken occludin in MDCK cells increased transepithelial electrical resistance. Their data are consistent with the idea that occludin expression can be a determinant of tight junction permeability. By northern blot analysis, occludin protein expression in the different types of endothelial cells appears to be regulated at the transcriptional level (Fig. 6B).

Chicken (Furuse et al., 1993) and mammalian epithelial (Saitou et al., 1997) occludins are detected as multiple bands by immunoblotting. Occludin in brain endothelial cells showed similar multiple bands. The molecular mass of the fastest-migrating band in lysates of both brain endothelial cells and LLC-PK$_1$ cells was very similar and about 58 kDa. Additional, more slowly migrating bands in brain endothelial cells were more abundant than in the LLC-PK$_1$ cells. These may represent alternatively spliced forms, as revealed by northern blot analysis, and/or posttranslationally modified protein, which may influence junctional properties. The minor bands seen in northern blots may account for at least two alternatively spliced forms (Fig. 6B). Similar results in northern blots of mRNA from mouse testis, kidney, liver, lung and brain have been obtained by Saitou et al. (1997).

With respect to the isoforms of ZO-1, Balda and Anderson (1993) reported that the α^+ isoform was expressed in most epithelial cells, while the α^- isoform was observed in endothelial cells as well as in well-developed junctions in Sertoli cells and glomerular epithelial cells. In our study, the immunoblot with an anti-ZO-1 antibody suggested a similar expression pattern for ZO-1 in endothelial cells from brain and LLC-PK$_1$ cells. However, only the faster-migrating band was detectable in aortic endothelial cells, raising two possibilities. One is that the relative ratio of ZO-1 α^+ and α^- isoforms in brain and aortic endothelial cells differs. The other is that posttranslational modifications of ZO-1 affecting mobility in SDS-PAGE are different in these two types of endothelial cells. Thus the content of the ZO-1 in brain endothelial cells is similar to that in the epithelial cells, suggesting that this may also be important to form tight junctions of low permeability. Also, ZO-1 has been reported to colocalize with the cadherin-catenin complex in fibroblasts and cardiac muscle cells which lack tight junctions (Itoh et al., 1991, 1993), as well as in MDCK cells during early stages in the assembly of tight junctions (Rajasekaran et al., 1996). Whether ZO-1 associates with occludin or cadherin in endothelial cells in non-neural tissues has yet to be determined.

Given that brain endothelial cells are characterized by high expression of occludin and that this may be essential for the formation of a continuous tight junction structure, we also examined the effects of known tight junction permeability modulators on occludin expression. The influence of ACM and CPT-cAMP, known inducers of the paracellular barrier (Rubin et al., 1991), and LPA, which impairs the barrier (Schulze et al., 1997), were investigated (Fig. 4E). However, occludin expression levels did not change in response to any of these modulators, which indicates that tight junction permeability in brain endothelial cells can be modulated without alteration of occludin expression.

Another interesting issue concerning endothelial tight junctions is the developmental timing of BBB formation. So far, despite many in vivo studies, when the BBB initially forms is still not clearly established. Also, developmental changes in the molecular composition of the BBB have not been fully investigated. Our results show that occludin expression in brain endothelial cells at P8 is extremely low (Fig. 7). This suggests that the phenotype of these cells at P8 resembles that of endothelial cells in non-neural tissue. In contrast, occludin is clearly expressed in brain endothelial cells at P70. It is therefore possible that occludin expression may determine the barrier phenotype of brain endothelial cells in vivo. Whether this barrier reflects limited permeability to larger molecules such as proteins or to smaller molecules such as ions remains to be determined. Also, mechanisms regulating occludin expression are as yet unknown, but will be intriguing issues for the future.

In conclusion, our experiments clearly demonstrate occludin expression in mammalian brain endothelial cells. In mature tissue, the expression level of occludin differs considerably depending on the source of the endothelial cells, being greater in those from brain than in those from non-neural tissue, and occludin expression may be transcriptionally regulated. Furthermore, occludin expression changes during the development of the BBB and this could be a crucial determinant of the unique junctional properties of this important endothelial barrier.

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